

Age-related Variation in Snake Venom: Evidence from Two Snakes (*Naja atra* and *Deinagkistrodon acutus*) in Southeastern China

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Abstract In this study we explored electrophoretic profiles, enzymatic activities and immunoreactivity of neonate and adult venoms from two snakes (*Naja atra* and *Deinagkistrodon acutus*) coexisting in southeastern China. Age-related variation in electrophoretic profiles was found in both species and proteolytic and fibrinogenolytic activity was higher in neonate than adult venoms. Neonate *D. acutus* venom had higher 5' nucleotidase, PLA₂, hyaluronidase and gelatinolytic activity, but lower esterolytic activity, than adult venom. Neonate and adult *D. acutus* venoms showed identical phosphomonoesterase, LAO and fibrinolytic activities. Neonate *N. atra* venom had higher phosphomonoesterase and LAO activity, but lower 5' nucleotidase, PLA₂, hyaluronidase and AchE activities than adult venom. Neonate and adult *N. atra* venoms showed similar gelatinolytic activity. Further, age-dependent immunoreactivity was found in both species, and cross-reactions between homologous venoms and antisera were closely related to venom composition. We speculate that age-related variation in venom characteristics is possibly driven by evolutionary forces associated with ontogenetic shifts in dietary habits, competition and predation pressure.

Keywords *Naja atra*, *Deinagkistrodon acutus*, Age-related variation, Electrophoretic profile, Enzymatic activity, Immunoreactivity

1. Introduction

Symptoms caused by venomous snake bites are closely associated with venom composition and activity (Chippaux, 1991) and toxinologists have long been intrigued by variation in the composition and enzymatic activity of snake venoms, including age-related variation between neonate and adult animals. However, in China, little attention has been paid to the symptomatic differences caused by envenomation by neonate or adult snakes and the potential divergence between symptoms has been largely ignored. We can often find that patients

bitten by neonate venomous snakes are unsuitably treated with adult antiserum without any detailed evaluation and analysis of the therapeutic effect, and this is largely because no antiserum has been developed to treat envenomation by neonate snakes. It has been reported for *Bothrops asper*, *Bothrops atrox*, *Crotalus atrox* and *Crotalus durissus durissus* that neonate venoms are more toxic (Gutiérrez *et al.*, 1980; Gutiérrez *et al.*, 1991; Minton and Weinstein, 1986; Saldarriaga *et al.*, 2003; Alape-Girón *et al.*, 2009). In some snake species of the genus *Bothrops* adult venoms show higher hydrolysis of casein, collagen, fibrinogen and gelatin (Furtado *et al.*, 1991; Antunes *et al.*, 2010; Zelanis *et al.*, 2010). Neonate snake venom may have a higher lethality in order to enhance foraging efficiency, whereas adult snakes strengthen their proteolytic activity to improve digestion (Hirth, 1966; Pough *et al.*, 1983). The neonate-

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to-adult transition of enzymatic activity and lethality has been detected in several species of venomous snakes (Mackessy *et al.*, 1988, 2006; Andrade *et al.*, 1999; but see also Mackessy *et al.*, 2003; Calvete *et al.*, 2010). This age-related variation in snake venoms presents a major challenge to antiserum preparation and the treatment of snakebites. For example, commercial antisera producing high titers for neutralizing adult venoms are less effective at neutralizing neonate venoms due to differences in composition and activity (Zelanis *et al.*, 2012; Antunes *et al.*, 2010; Saravia *et al.*, 2002).

Studies on age-related variation in snake venoms have mainly focused on pit vipers of the genera *Crotalus*, *Bothrops*, *Lachesis* and *Gloydius* (Mackessy, 1988; Furtado *et al.*, 1991; Zelanis *et al.*, 2011; Madrigal *et al.*, 2012; Durban *et al.*, 2013; Gao *et al.*, 2013a, 2014), and have confirmed significant divergence between neonate and adult venoms (Maruyama *et al.*, 1990). In envenomation by *Bothrops jararaca* and *B. moojeni*, for example, neonate snakes cause higher coagulating activity than adults, while bites from adult snakes result in a higher incidence of local tissue necrosis than from neonates (Kouyoumdjian and Polyzelli, 1989; Ribeiro and Jorge, 1989).

The Chinese cobra *Naja atra* and the five-paced pit viper *Deinagkistrodon acutus* are among the five most actively traded venomous snakes in China and account for a significant proportion of human and domesticated animal envenomation (Qin, 1998; Zhao, 2006). The two snakes coexist south of the Yangtze River and inhabit diverse environments including human-dwellings (Qin, 1998; Zhao, 2006). In Tiantai, Zhejiang for example, snakebites by these two species accounted for 13% of total snakebites in 2007 and 23% in 2010 (Wang *et al.*, 2011). Victims bitten by *N. atra* usually suffer from edema, necrosis, tremors, blurred vision, tachypnea and arrhythmia, with the wounds seldom bleeding. Victims may also suffer from acute respiratory, circulatory and renal failure. Victims bitten by *D. acutus* can suffer from edema, ecchymoses, necrosis, angina, blurred vision, hematuria and arrhythmia, and their blood pressure may drop drastically because of excessive bleeding. Acute circulatory failure, acute renal failure, and cerebral hemorrhages occur in seriously injured patients.

Given the high frequency of *N. atra* and *D. acutus* bites, the severe and wide-ranging effects of their venoms, and patterns of age-related variation in venoms observed in other species, there is a need to explore neonate and adult venoms in these two important species and improve treatments and antiserum production.

However, before improvements to treatments can be developed, fundamental characteristics of neonate and adult venoms in these species must be described. In this study we measured the electrophoretic profiles, enzymatic activity and immunological reactivity of neonate and adult venoms collected from *N. atra* and *D. acutus*. Our objective was to broaden the understanding of age-related variation in snake venoms and provide a foundation for subsequent research aimed at improving the efficacy of snake bite treatments in China.

2. Materials and Methods

2.1 Snakes and venoms We collected adult *N. atra* from Guangxi, China and adult *D. acutus* from Zhejiang, China in late June 2012. Snakes were maintained in our laboratory in Hangzhou, where females laid eggs between mid-July and early August. Eggs were incubated at 24–28 °C until hatching. Adult venoms (22 *N. atra* and 31 *D. acutus*) were milked by biting on a parafilm-wrapped jar, and neonate (2–3 weeks old) venoms (15 *N. atra* and 10 *D. acutus*) were collected using pipette micro tips, and each snake was milked only once. Fresh venoms were pooled by species and age, and then centrifuged to remove impurities for 15 min at 10 000 g 4 °C, lyophilized and stored at –80 °C until use. Commercial monospecific antisera were purchased from Shanghai Serum Biological Technology. Venom protein was determined according to Bradford (1976) with BSA as the standard. In this study, we adhered to the Wild Animals Protection Law of the People's Republic of China. All experiments involving live snakes were approved by the Animal Ethics Committee at Hangzhou Normal University.

2.2 The separation of venom protein by 1-DE and 2-DE Both neonate and adult venoms were separated by 1-DE according to Laemmli (1970). Samples were applied to 3% stacking gel and 12.5% separation gels under reducing and non-reducing conditions. The gels were stained in 0.2% Coomassie Brilliant Blue R-250, and destained with 10% acetic acid in water/methanol (v/v = 1:1).

Prior to the separation of venom protein by 2-DE, venom samples were precipitated by pre-cooled acetone, and centrifuged at 13 000 g 4 °C for 30 min. The precipitates were washed and dried, and then redissolved in rehydration solution [8 M urea, 4% CHAPS, 65 mM DTT, 0.2% pharmalyte (pH 3–10) and 0.002% bromophenol blue]. One hundred and fifty micrograms of protein was loaded on 7 cm IPG precast strips (pH 3–10), and isoelectric focused at 20 °C according to the

following steps: 300 V for 3 h, 500 V for 1 h, 1 000 V for 1 h, 4 000 V for 3 h and 4 000 V for 20 000 V·h. The strips were then reduced and alkylated by sequential incubation with 2% DTT and 2.5% iodoacetamide in equilibration buffer (6 M urea, 1.5 M Tris-HCl, 20% glycerol, 2% SDS, 0.002% bromophenol blue, pH 8.8), and applied to 12.5% SDS-PAGE gels for the second dimension separation. The gels were stained and destained as described above. Results were scanned using a UMax2100 densitometer (Umax Technologies). CHAPS, pharmalyte, bromophenol blue, iodoacetamide and IPG strips were purchased from Bio-Rad Laboratories, Inc, and other regants were purchased from Sangon Biotech Co., Ltd. The experiment procedure was carried out on the system from Bio-Rad Laboratories, Inc.

2.3 Enzymatic activities Enzymatic activities were assayed according to Gao (2010) and Gao *et al.* (2011). Proteolytic activity was evaluated using bovine casein and human hemoglobin as substrates with L-Tyrosine as the standard; the unit of enzymatic activity within 2 h at 37 °C was defined as nmol of L-Tyrosine released/min/mg venom protein. Arginine esterolytic activity was carried out using chemical synthetic substrates, and the unit of enzymatic activity was defined as nmol of *p*-nitroaniline (for BAPNA) released or nmol of the substrate (for TAME) degraded min/mg venom protein. 5' nucleotidase activity was assayed using 5' AMP as the substrate, and KH_2PO_4 was used as the standard, with activity defined as nmol of inorganic phosphate released min/mg venom protein. Phosphomonoesterase activity was assayed using *p*-nitrophenyl phosphate as the substrate, and *p*-nitrophenyl was used as the standard, with the unit of enzymatic activity defined as nmol of *p*-nitrophenyl released min/mg venom protein. LAO (L-amino acid oxidase) activity was assayed using L-Leucine as the substrate, and H_2O_2 was used as the standard, with the enzymatic activity defined as nmol of H_2O_2 degraded/min/mg venom protein. PLA_2 (Phospholipase A_2) activity was assayed using soybean lecithin as the substrate, and the unit of enzymatic activity that in one min of one mg venom protein was defined as increase in absorbance of 0.3. Hyaluronidase activity was determined using human hyaluronic acid as the substrate, and hyaluronidase with high purity was used as the standard, the activity was expressed as national formulary units (NFU)/min/mg venom protein. For the assay of fibrinogenolytic activity, human plasma fibrinogen was used as the substrate and incubated with venom for 4 min at 37 °C, and the degradation characteristics were determined using SDS-PAGE on 7.5% polyacrylamide gel according to the

method described above. The AchE (acetylcholinesterase) activity was determined by hydrolyzing acetylcholine iodide, and the unit of activity that in one min of one μg venom protein was expressed as increase in absorbance (412 nm) of 0.001. Fibrinolytic activity was assayed using bovine plasminogen-rich fibrinogen as substrate, and the enzymatic activity was defined as mm^2 of the clear area formed/ μg venom protein. For gelatin zymography assay, the venoms were separated under non-reducing condition on 12% polyacrylamide gel copolymerized with gelatin. After electrophoresis, the gel was treated with renaturing buffer at 37 °C for 16 h, and then stained by Coomassie Brilliant Blue R-250. Gelatinolytic activity was indicated by clear zones present on the gel.

2.4 Western blotting Immunological reactions between venoms and antisera were done using western blotting and following Gao *et al.* (2013b). After separation by 12% SDS-polyacrylamide gels, venoms were transferred to 0.45 μm PVDF membranes (millipore) in a semi-dry system (Bio-Rad). The membranes were then blocked with a buffer system (2% nonfat milk powder in 0.01 M PBS, pH 7.4) at 4 °C over night. After, membranes were washed with 0.01 M PBS pH 7.4, and incubated with commercial antisera diluted 1: 1000 at 37 °C for 1 h. Each membrane was then washed and incubated with AP-conjugated anti-horse IgG diluted 1: 3000 at 37 °C for 1 h. The color reaction was developed with substrate solution (0.15 mg/ml BCIP and 0.3 mg/ml NBT in 0.1 M Tris-HCl, pH 9.5, containing 50 mM MgCl_2 and 0.1 M NaCl). Results were then scanned using a UMax2100 densitometer (Umax Technologies).

3. Results and Discussion

3.1 Variation in the electrophoretic profiles of venom composition Regardless of the application of 1-DE or 2-DE, venom composition differed between neonates and adults in both species. In *N. atra* we found that: (1) the number of protein bands detected in neonate and adult venoms were similar under non-reducing conditions (Figure 1 A), with one exception of a band with molecular mass of ~182 kDa; (2) a band with molecular mass of ~160 kDa was present in both venoms but much brighter in adult venom; (3) neonate venom had a higher amount in the region with molecular masses of ~49–86 kDa, but a lower amount in the region with molecular masses of ~19 kDa; (4) protein bands with molecular masses higher than ~116 kDa could not be found in neonate or adult venom under reducing conditions (Figure 1 B), whereas several new bands with molecular masses of ~115, ~47, ~32 and

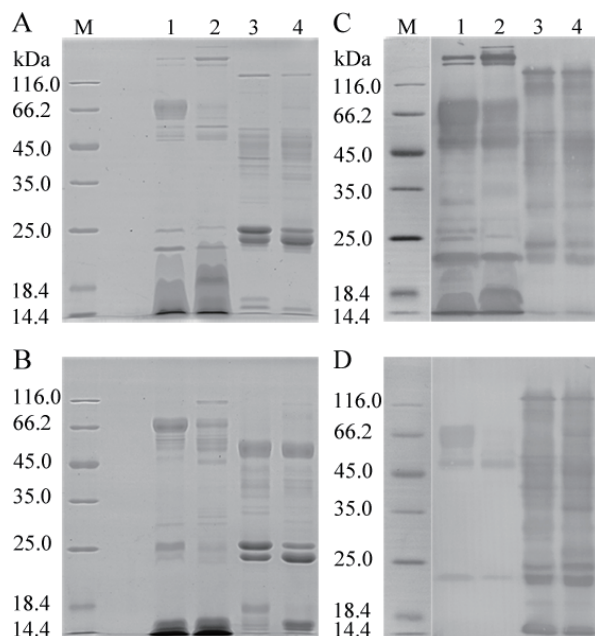


Figure 1 Electrophoretic profiles and western blots of pooled neonate and adult venoms. Six micrograms of protein was loaded on each lane, and then separated on 12.5% gels under non-reducing (A, C, D) and reducing (B) conditions. Electrophoretic profiles were stained by Coomassie brilliant blue (A, B). Western blot of commercial antisera against the venoms: *N. atra* antiserum (C) and *D. acutus* antiserum (D). Venoms: neonate (lane 1, 3) and adult (lane 2, 4), *N. atra* (lane 1, 2) and *D. acutus* (lane 3, 4).

~31 kDa were found, with the first three bands found only in adult venom; and (5) the component region with molecular masses of ~14–16 kDa became thicker than under non-reducing conditions.

In *D. acutus*, neonate and adult venoms showed similar electrophoretic profiles with some slight distinctions (Figure 1 A). There were three protein bands with molecular masses of ~98 (very weak), ~31 and ~17 kDa preferentially presented in neonate venom, and two bands with molecular masses of ~66 and ~36 kDa only presented in adult venom. Additionally, four protein bands with molecular masses of ~130, ~56, ~42, and ~25 kDa were more abundant in neonate venom, and one band with molecular mass of ~24 kDa was more abundant in adult venom. Under reducing conditions the protein bands with molecular mass of ~130 kDa disappeared in both venoms, and some of the components distributed across ~35–45 kDa were degraded (Figure 1 B). A new protein band with molecular mass of ~18 kDa was found in neonate venom, and the intensity of components around ~16 kDa was higher in adult venom. The disappearance of components with high molecular masses indicates that these components are multimeric proteins, whereas the

increased components with low molecular masses are the subunits of these multimeric proteins.

Differences in composition between neonate and adult venoms have been detected by 2-DE in elapid and viperid snakes (Li *et al.*, 2004; Guércio *et al.*, 2006; Gao *et al.*, 2013a). Previous studies have shown that the main components of venoms from elapid snakes belong to basic proteins with small molecular masses (Nawarak *et al.*, 2003). Here, the venom composition of *N. atra* presented the same profile, with neonate venom showing higher intensity in the area of middle and high molecular masses (Figure 2). Compared with neonate venom, adult venom expressed extra proteins with molecular masses/pI of ~115 kDa/8.0–8.8 and ~17 kDa/5.7. Neonate venom had a higher abundance of components with molecular masses/pI of ~66–89 kDa/5.7–7.6 and ~15 kDa/7.4–7.8, while adult venom had a higher abundance of components with molecular masses/pI of ~59–61 kDa/6.7–8.3 and ~48 kDa/5.5.

Similar to the electrophoretic profile of *D. acutus* venom explored by Huang *et al.*, (2009), venom proteins in both neonate and adult *D. acutus* were distributed equally in the gels according to the pI (Figure 2). Proteins with molecular masses/pI of ~44–52 kDa/3.6–4.7 were specifically expressed in neonate venom, and proteins with molecular masses/pI of ~88–98 kDa/4.1–4.9 and ~104 kDa/5.6–6.0 were found in adult venom. Proteins with molecular masses/pI of ~44–45 kDa/5.3–5.6 and ~26–27 kDa/7.4–9.5 were more highly expressed, and those with molecular masses/pI of ~25 kDa/3.0–6.0 and below ~18 kDa were less expressed in neonate venom. The difference in venom composition between neonate and adult snakes may be influenced by many factors such as chemical modification via glycosylation (Gao *et al.*, 2013a).

3.2 Enzymatic activity Neonate venoms expressed higher proteolytic activity in hydrolyzing casein and hemoglobin than adult venoms for both *N. atra* and *D. acutus* (Table 1). All venoms could hydrolyze the Aa chain of human fibrinogen; fibrinogenolytic activity was higher in neonate venoms in both species, and was higher in *D. acutus* venom (Figure 3). These results suggest that fibrinogenolytic components (e.g. metalloproteinases) are more active in neonate venom than adult venom, and are more active in *D. acutus* venom than *N. atra* venom. Gelatin zymography indicated that the clearance area with molecular mass of ~100 kDa hydrolyzed by neonate and adult *N. atra* venoms was very weak (Figure 4). No age-related difference in gelatinolytic activity is apparent and this may be because venoms secreted by

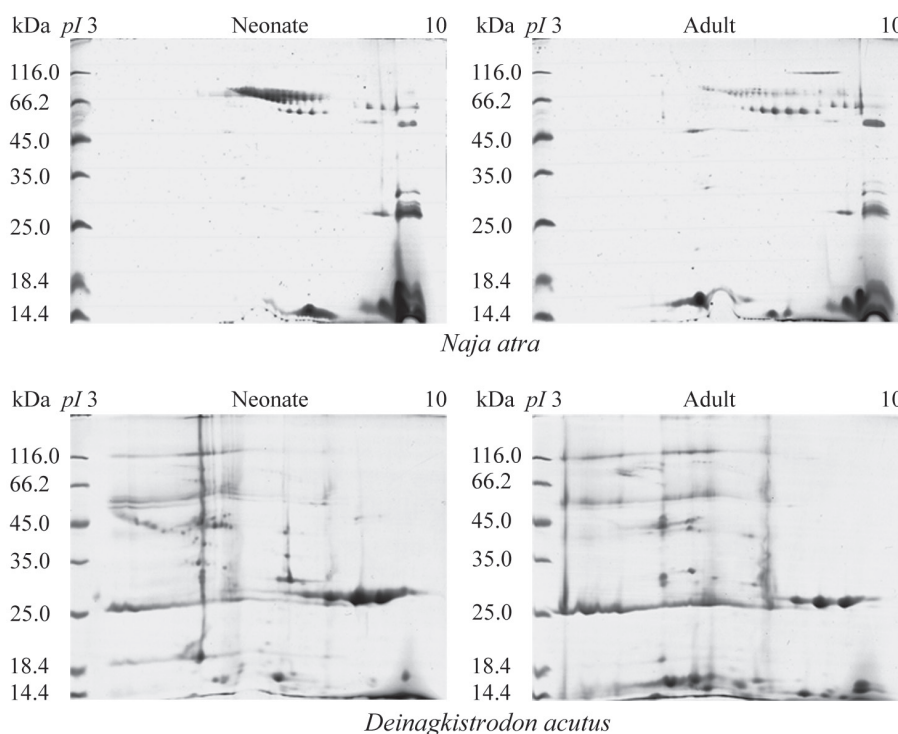


Figure 2 Comparative analysis of two-dimensional gel electrophoresis profiles of neonate and adult venoms. Pooled venom (150 μ g protein) from neonate or adult snakes was applied to IPG precast strips (pH 3-10L, 7 cm) followed by electrophoresis on 12.5 % SDS-polyacrylamide gels.

elapid snakes always contain numerous neurotoxins and a few metalloproteinases (Tan and Ponnudurai, 1990; Li *et al.*, 2004; Fernández *et al.*, 2011; Rey-Suárez *et al.*, 2011). In *D. acutus*, an apparent clearance zone with molecular masses of ~29–45 kDa and ~24 kDa is induced by neonate venom, which is much brighter than that induced by adult venom (Figure 4). Age-related variation in gelatinolytic activity of *D. acutus* venoms indicates that the metalloproteinase and serine proteinase with gelatinolytic activity may be expressed more abundantly in neonate venom. Compared with *N. atra* venoms, *D. acutus* venoms display higher abundance of metalloproteinase and serine proteinase with gelatinolytic activity. High amounts of neurotoxins are incompatible with high proteolytic activity (Mackessy *et al.*, 2003), as is the case in *N. atra* where adult venom shows higher PLA₂ and AchE activity and higher lethality than neonate venom. In *D. acutus*, however, neonate venom has higher PLA₂ activity than adult venom, and may also induce higher lethality. The above results suggest that neonate *N. atra* venom may express a higher digestion activity and lower foraging efficiency than adult venom. Moreover, it seems likely that high proteolytic activity and lethality are compatible in neonate *D. acutus* venom. Higher competition or predation pressure may have been

imposed on adult *N. atra* venom, which has evolved greater toxicity than that of neonate venom. In contrast, the competition or predation pressure may be weaker in *D. acutus* and, as such, its venom has evolved to adapt to ontogenetic shifts in diet.

Adult *D. acutus* venom was more active than neonate venom regarding esterolytic activity (Table 1), suggesting more abundant venom components with serine proteinase activity in adult venom. Adult *N. atra* venom was 1.1 and 5.2 times more active than neonate venom in 5' nucleotidase and hyaluronidase activities, respectively (Table 1). This suggests that the potential blood coagulant and venom permeability are more active in adult venom. In contrast, neonate *D. acutus* venom was respectively 2.0 and 1.1 times more active than adult venom for these two activities. Neonate *N. atra* venom was 4.0 and 1.3 times more active than adult venom in phosphomonoesterase and LAO activities, respectively; no such age-related variation was found in *D. acutus* venoms. There was almost no age-related variation in the cleaved area on the fibrin-plate caused by *D. acutus* venom (Table 1), suggesting that neonate and adult *D. acutus* venoms contain almost identical amounts of fibrinolytic components. Further, *N. atra* venom showed no esterolytic or fibrinolytic activity, whereas *D. acutus*

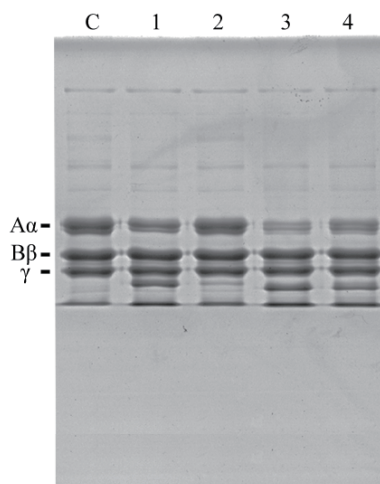


Figure 3 Fibrinogenolytic activity of pooled neonate and adult venoms. The fibrinogen was separated on 7.5 % SDS-polyacrylamide gel. Venoms: Neonate (lanes 1, 3) and adult (lanes 2, 4), *N. atra* (lanes 1, 2) and *D. acutus* (lanes 3, 4). C: Control of fibrinogen incubated without venom. Aα, Bβ, γ indicate three chains of fibrinogen.

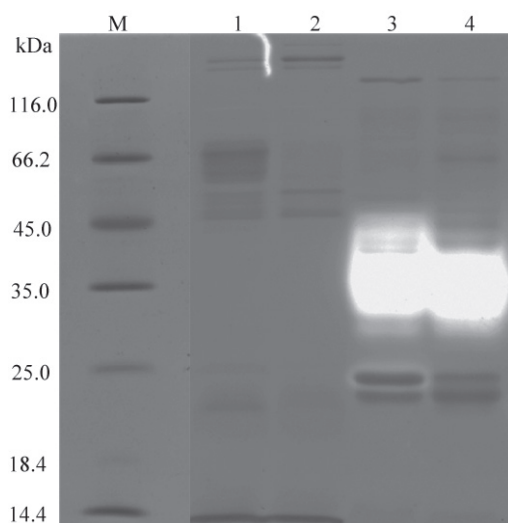


Figure 4 Gelatinolytic activity of pooled neonate and adult venoms evaluated by zymography. The venoms were separated on 12 % SDS-polyacrylamide gel copolymerized with 0.2 % gelatin. Venoms: Neonate (lanes 1, 3) and adult (lanes 2, 4), *N. atra* (lanes 1, 2) and *D. acutus* (lanes 3, 4).

venom showed no AchE activity.

3.3 Immunoreactivity between venoms and antisera

The commercial antiserum for adult venom is often raised in horses; neonate venom is generally ignored. In China, only four commercial monovalent antisera have been raised against *Bungarus multicinctus*, *N. atra*, *D. acutus* and *Gloydius brevicaudus* venoms. Here, we used two commercial monovalent antisera to evaluate the

immunoreactivity of neonate and adult venoms of *N. atra* and *D. acutus* by western blotting. We found that cross-reactions between homologous venoms and antisera were closely associated with venom composition, and reactions were stronger between homologous than heterologous venoms and antisera (Figure 1 C–D). Cross-reactions showed several differences between neonate and adult venoms. Compared with adult venom of the same species, neonate *N. atra* venom showed weaker or even no intensity in three protein bands with molecular masses of ~33, ~29 and ~27 kDa, but expressed higher intensity in the area with molecular masses of ~49–86 kDa. Adult *N. atra* venom presented a special blotting band with molecular mass of ~182 kDa, and showed higher intensity in the band with molecular mass of ~160 kDa than neonate venom. The cross-reactivity between *N. atra* venom and *D. acutus* antiserum was weak, and only identified in the region with molecular masses of ~51–82 kDa and ~23 kDa. Neonate *N. atra* expressed higher reactivity in the ~60–82 kDa region as compared with adult venom. In reactions with *D. acutus* antiserum, *D. acutus* venom showed age-related differences in immunoreactivity (Figure 1 D). The cross-reacting bands with molecular masses of ~66, ~35 and ~27 kDa were evidently detected in adult *D. acutus* venom, and the ~20 kDa and the area around ~30 kDa were identified in neonate venom. In contrast, in reactions with *N. atra* antiserum, neonate and adult *D. acutus* venoms showed similar immunoreactivities, and the cross-reaction occurred in the areas with molecular masses of ~100–130, ~31–57 kDa and ~24 and ~23 kDa. Undoubtedly, this difference in immunological intensity and the presence of age-specific bands between neonate and adult venoms can be attributed to age-related differences in venom composition.

Age-related variation in immunoreactivity has been reported for many venomous snakes, and some studies have declared that commercial antisera are less effective in neutralizing neonate venoms (Kamiguti *et al.*, 1988; Maruyama *et al.*, 1990; Antunes *et al.*, 2010). Higher doses of antiserum should be used to treat patients bitten by neonate snakes if the same amount of venom were theoretically injected by both venoms (Kamiguti *et al.*, 1988; Antunes *et al.*, 2010). The results of this study suggest that differences between neonate and adult venoms should be considered when treating patients bitten by *N. atra* and *D. acutus*. To reduce allergic reaction to over injection of antiserum, we should improve the preparation of antiserum by first distinguishing adult venom versus neonate venom. The capacity of *D. acutus*

Table 1 Enzymatic activity of pooled venoms from neonate and adult snakes.

Enzymatic activities Substrates	<i>Naja atra</i>			<i>Deinagkistrodon acutus</i>		
	Neonates	Adults	Statistical results	Neonates	Adults	Statistical results
Proteolytic activity						
Casein (nM/min/mg)	14.4 ± 0.2	4.6 ± 0.1	$P < 0.0001$, N > A	106.0 ± 1.2	96.3 ± 0.8	$P < 0.01$, N > A
Hemoglobin (nM/min/mg)	3.5 ± 0.1	1.8 ± 0.1	$P < 0.0002$, N > A	15.4 ± 0.1	13.6 ± 0.1	$P < 0.0002$, N > A
Esterolytic activity						
BAPNA (nM/min/mg)	-	-		3.9 ± 0.2	4.8 ± 0.4	$P < 0.05$, N < A
TAME (nM/min/mg)	-	-		110.0 ± 2.3	147.4 ± 1.7	$P < 0.0002$, N < A
5' nucleotidase activity						
AMP (nM/min/mg)	2992.7 ± 56.8	3321.3 ± 85.7	$P < 0.05$, N < A	426.1 ± 12.0	206.8 ± 6.2	$P < 0.0001$, N > A
Phosphomonoesterase activity						
pNPP-Na (nM/min/mg)	84.9 ± 1.0	21.9 ± 0.4	$P < 0.0001$, N > A	0.3	0.3	N = A
PLA ₂ activity						
Soybean lecithin (U/min/mg)	160.2 ± 7.6	1291.6 ± 27.2	$P < 0.0001$, N < A	880.5 ± 33.8	63.8 ± 3.3	$P < 0.0001$, N > A
Hyaluronidase activity						
Hyaluronic acid (NFU/min/mg)	0.5 ± 0.1	2.6 ± 0.1	$P < 0.0001$, N < A	5.3	5.0 ± 0.1	N = A
L-amino oxidase activity						
L-Leu (nM/min/mg)	31.6 ± 0.1	24.4 ± 0.2	$P < 0.0001$, N > A	0.3	0.3	N = A
Acetylcholinesterase activity						
Acetylcholine iodide (U/min/μg)	9.5 ± 1.9	71.8 ± 1.0	$P < 0.0001$, N < A	-	-	
Fibrinolytic activity						
Fibrin (mm ² /μg)	-	-		20.9 ± 0.4	20.6 ± 0.4	$P = 0.66$, N = A

Data are expressed as mean ± standard error.

-: no activity. N: neonate; A: adult.

antiserum to neutralize *N. atra* venoms is weak, so is the capacity of *N. atra* antiserum to *D. acutus* venoms. Thus, patients bitten by *N. atra* should not be treated with *D. acutus* antiserum, and vice versa.

In conclusion, our data show age-related variation in venom composition, enzymatic activity and immunoreactivity in *N. atra* and *D. acutus*. Variation in enzymatic activity and immunoreactivity is associated with variability in venom composition. Age-dependent variation in venom characteristics is possibly driven by evolutionary forces associated with ontogenetic shifts in dietary habit, competition and predation pressure. The results of this study will hopefully stimulate further research into age-related variation in snake venoms and provide a foundation for future research aimed at improving the efficacy of snake bite treatments.

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